invitrogen™

pcDNA™4/HisMax A, B, and C

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User Manual

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Kit Contents and Storage

Shipping and Storage

pcDNA™4/HisMax vectors are shipped on wet ice. Upon receipt, store vectors at -20°C.

Kit Contents

All vectors are supplied as detailed below. **Store the vectors at –20°C.**

Vector	Composition	Amount
pcDNA™4/HisMax A, B, and C	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
pcDNA™4/HisMax/*lacZ*	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg

Introduction

Product Overview

Description of the System

pcDNA™4/HisMax A, B, and C are 5.3 kb vectors derived from pcDNA™4/His and designed for overproduction of recombinant proteins in mammalian cell lines. Features of the vectors allow purification and detection of expressed proteins (see pages 13-14 for more information). High-level stable and transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells.
- QBI SP163 translational enhancer for increased levels of recombinant protein expression (Stein et al., 1998) (see page 3 for more information).
- Three reading frames to facilitate in-frame cloning with an N-terminal peptide encoding the Xpress™ epitope and a polyhistidine metal-binding tag.
- Zeocin™ resistance gene for selection of stable cell lines (Mulsant et al., 1988) (see page 16 for more information).
- Episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-1, COS-7).

The control plasmid, pcDNA™4/HisMax */lacZ*, is included for use as a positive control for transfection, expression, and detection in the cell line of choice.

Experimental Outline

Use the following outline to clone and express your gene of interest in pcDNA™4/HisMax.

Consult the multiple cloning sites described on pages 4-6 to determine which vector (A, B, or C) should be used to clone your gene in frame with the N-terminal Xpress™ epitope and the polyhistidine tag.

1. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on 50 to 100 µg/mL ampicillin or 25-50 µg/mL Zeocin™.
2. Analyze your transformants for the presence of insert by restriction digestion.
3. Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in frame with the N-terminal peptide.
4. Transfect your construct into the cell line of choice using your own method of transfection. Generate a stable cell line, if desired.
5. Test for expression of your recombinant gene by western blot analysis or functional assay. For the antibody to the Xpress™ epitope, see page 19.

To purify your recombinant protein, you may use metal-chelating resin such as ProBond™. ProBond™ resin is available separately (see page 19 for ordering information).

Methods

Cloning into pcDNA™4/HisMax A, B, and C

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al*., 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al*., 1994).

E. coli Host

Many *E. coli* strains are suitable for the propagation of this vector. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A). For your convenience, TOP10F´ is available as chemically competent or electrocompetent cells from Invitrogen (see page 19).

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintaining pcDNA™4/HisMax

To propagate and maintain the pcDNA™4/HisMax vectors, use a small amount of the supplied 0.5 µg/µL stock solution in TE, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like TOP10F´, DH5α, JM109, or equivalent. Select transformants on LB plates containing 50 to 100 µg/mL ampicillin or 25 to 50 µg/mL Zeocin™ in Low Salt LB. Be sure to prepare a glycerol stock of each plasmid for long-term storage (see page 7 for protocol).

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Cloning into pcDNA™4/HisMax A, B, and C, Continued

QBI SP163 Translational Enhancer

The QBI SP163 element is a 163 nucleotide splice variant derived from the 5´ untranslated region (UTR) of the mouse vascular endothelial growth factor (VEGF) gene (Stein *et al.*, 1998). The splice variant is composed of a 31 nucleotide fragment containing the 5´ cap sequence of the VEGF gene fused to a 132 nucleotide fragment of the 5´ UTR immediately preceding the translational start site of the VEGF gene. Refer to the diagrams on pages 4-6 for the sequence of the QBI SP163 element.

The QBI SP163 element functions as a strong translational enhancer and acts to increase recombinant protein production when placed directly upstream of the ATG initiation codon of the gene of interest. The increase in protein expression is thought to occur through ribosome recruitment and a cap-independent translation mechanism. (Stein *et al.*, 1998).

In general, expression levels of recombinant protein from pcDNA™4/HisMax are 2-5 fold greater than the levels obtained with the pcDNA™4/His expression vector. The amount of recombinant protein expressed will vary depending on the nature of the gene of interest.

Note

The pcDNA™4/HisMax vectors are fusion vectors. To ensure proper expression of your recombinant protein, you must clone your gene in frame with the ATG at base pairs 1080–1082. This creates a fusion with the N-terminal polyhistidine tag, Xpress™ epitope, and the enterokinase cleavage site. The vector is supplied with the multiple cloning site in three reading frames relative to the N-terminal peptide to facilitate cloning. See pages 4-6 to develop a cloning strategy.

If you wish to clone your gene as close as possible to the enterokinase cleavage site, follow the guidelines below:

- Digest pcDNA™4/HisMax A, B, or C with *Kpn* I.
- Create blunt ends with T4 DNA polymerase and dNTPs.
- Clone your blunt-ended insert in frame with the lysine codon (AAG) of the enterokinase recognition site.

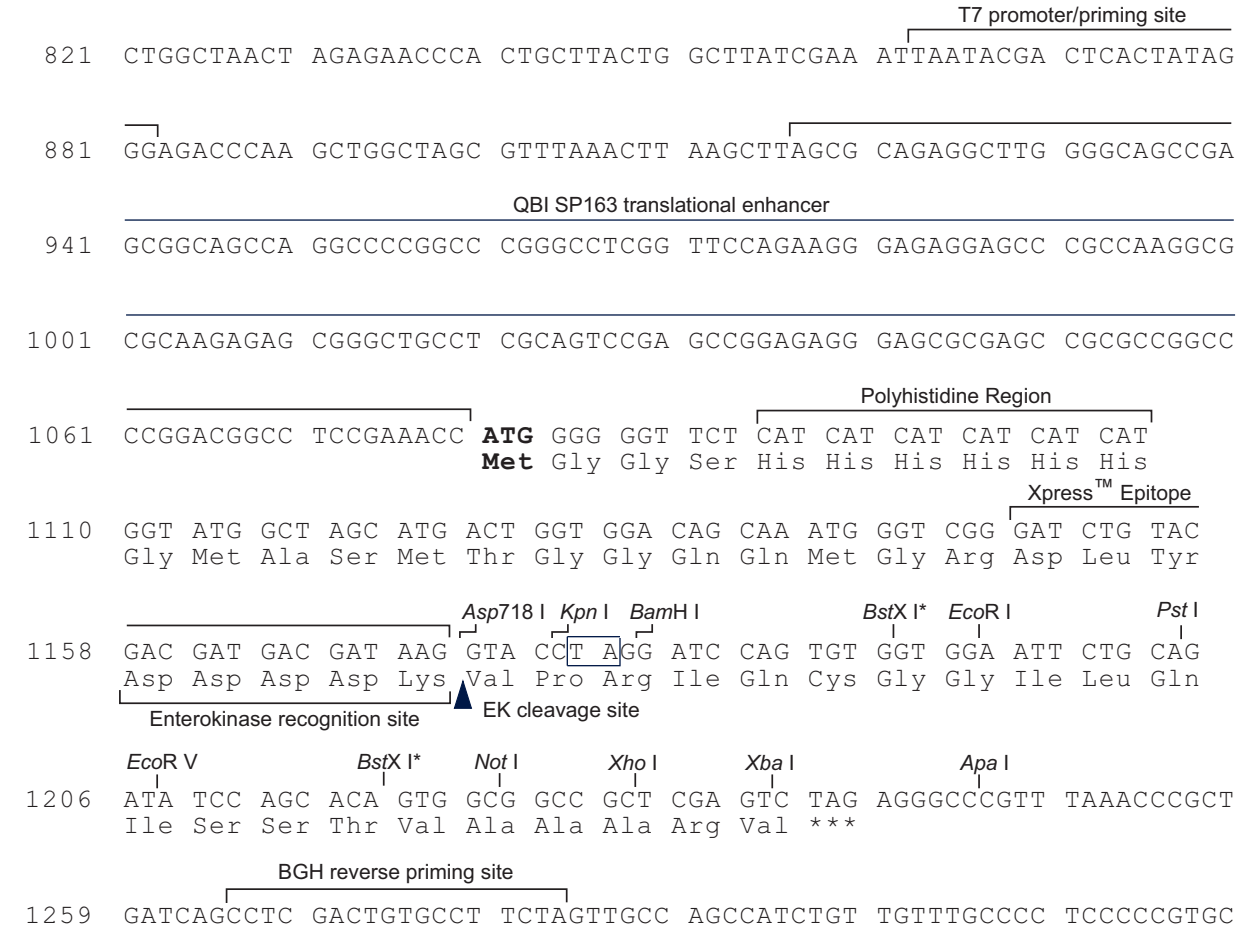
If you wish to separate your protein of interest from the N-terminal peptide tag, you may use any suitable enterokinase including EnterokinaseMax™ from Invitrogen (see page 19). Following enterokinase cleavage, no vector-encoded amino acid residues will be present in your protein.

Continued on next page

Cloning into pcDNA™4/HisMax A, B, and C, Continued

Multiple Cloning Site of Version A

Below is the multiple cloning site for pcDNA™4/HisMax A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. **Note that there is a stop codon within the *Xba* I site.** The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pcDNA™4/HisMax A is available for downloading from our website (www.invitrogen.com) or from **Technical Support** (see page 20).

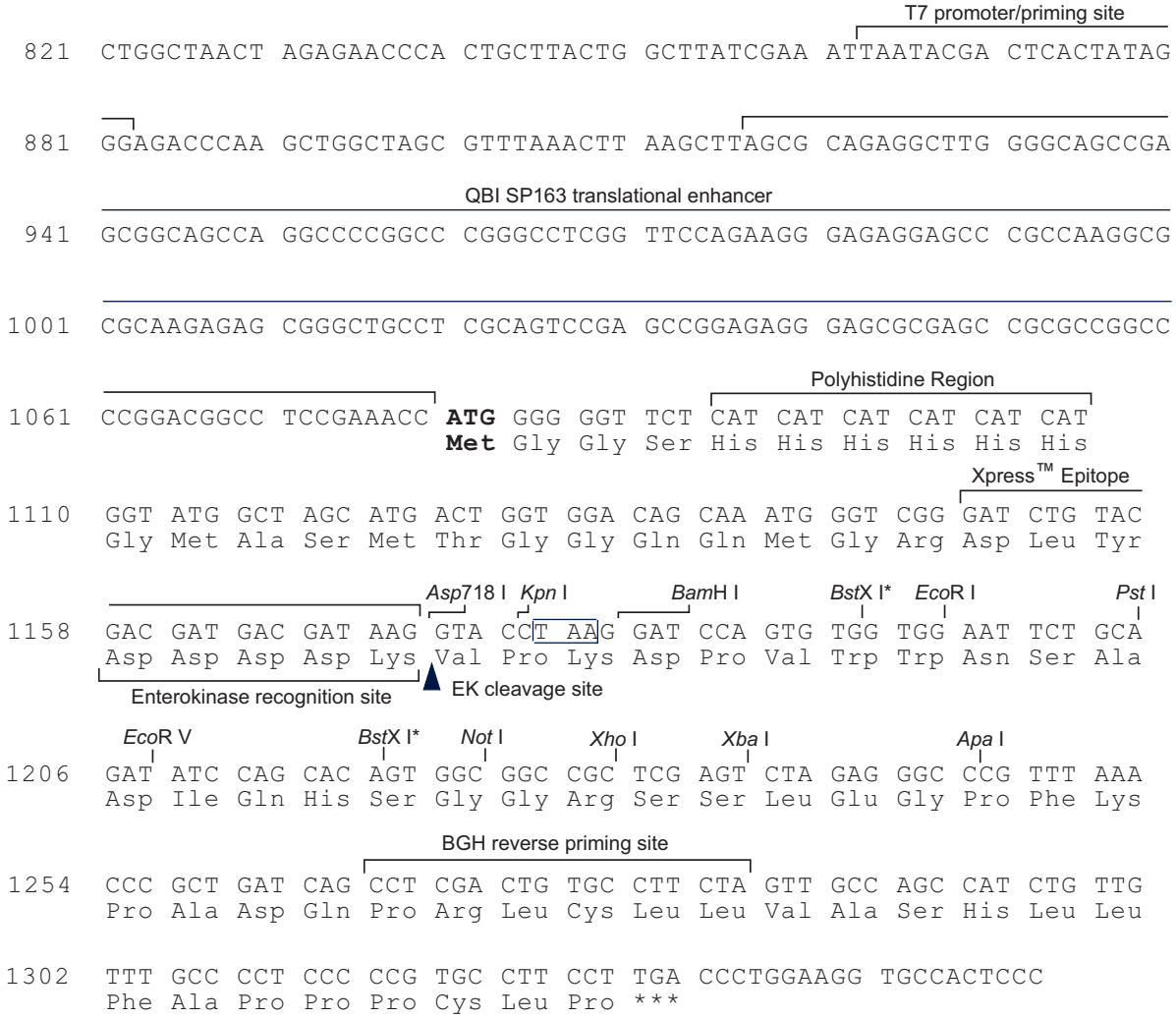

T7 promoter/priming site
821 CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG
881 GGAGACCCAA GCTGGCTAGC GTTTAAACTT AAGCTTAGCG CAGAGGCTTG GGGCAGCCGA
QBI SP163 translational enhancer
941 GCGGCAGCCA GGCCCCGGCC CGGGCCTCGG TTCCAGAAGG GAGAGGAGCC CGCCAAGGCG
1001 CGCAAGAGAG CGGGCTGCCT CGCAGTCCGA GCCGGAGAGG GAGCGCGAGC CGCGCCGGCC
Polyhistidine Region
1061 CCGGACGGCC TCCGAAACC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT
Met Gly Gly Ser His His His His His His
Xpress™ Epitope
1110 GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC
Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr
Asp718 I Kpn I BamH I BstX I* EcoR I Pst I
1158 GAC GAT GAC GAT AAG GTA CCT AGG ATC CAG TGT GGT GGA ATT CTG CAG
Asp Asp Asp Asp Lys Val Pro Arg Ile Gln Cys Gly Gly Ile Leu Gln
Enterokinase recognition site
EK cleavage site
EcoR V BstX I* Not I Xho I Xba I Apa I
1206 ATA TCC AGC ACA GTG GCG GCC GCT CGA GTC TAG AGGGCCCGTT TAAACCCGCT
Ile Ser Ser Thr Val Ala Ala Ala Arg Val ***
BGH reverse priming site
1259 GATCAGCCTC GACTGTGCCT TCTAGTTGCC AGCCATCTGT TGTTTGCCCC TCCCCCGTGC

Continued on next page

Cloning into pcDNA™4/HisMax A, B, and C, Continued

Multiple Cloning Site of Version B

Below is the multiple cloning site for pcDNA™4/HisMax B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pcDNA™4/HisMax B is available for downloading from our website (www.invitrogen.com) or from **Technical Support** (see page 20).

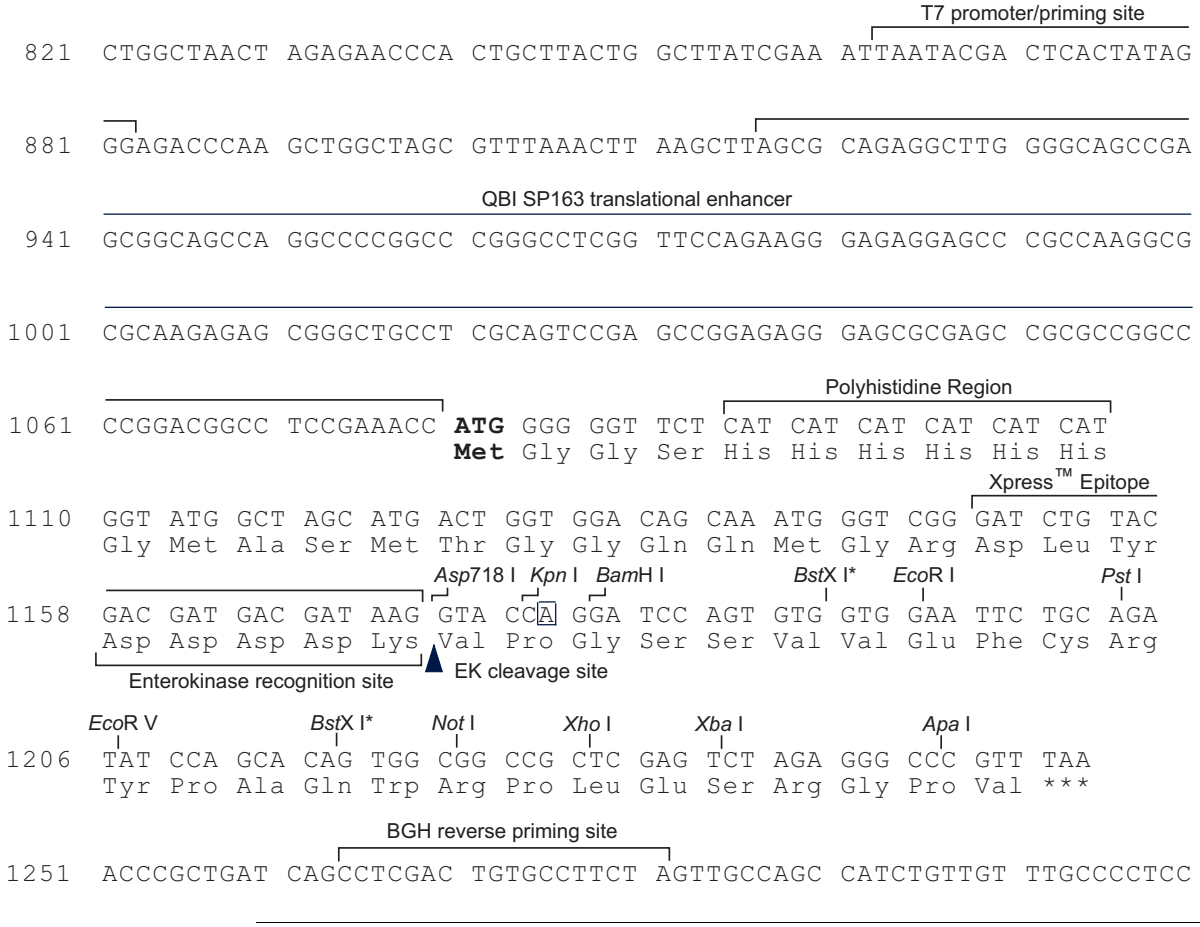

T7 promoter/priming site
821 CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG
881 GGAGACCCAA GCTGGCTAGC GTTTAAACTT AAGCTTAGCG CAGAGGCTTG GGGCAGCCGA
QBI SP163 translational enhancer
941 GCGGCAGCCA GGCCCCGGCC CGGGCCTCGG TTCCAGAAGG GAGAGGAGCC CGCCAAGGCG
1001 CGCAAGAGAG CGGGCTGCCT CGCAGTCCGA GCCGGAGAGG GAGCGCGAGC CGCGCCGGCC
Polyhistidine Region
1061 CCGGACGGCC TCCGAAACC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT
Met Gly Gly Ser His His His His His His
Xpress™ Epitope
1110 GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC
Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr
Asp718 I Kpn I BamH I BstX I* EcoR I Pst I
1158 GAC GAT GAC GAT AAG GTA CCT AAG GAT CCA GTG TGG TGG AAT TCT GCA
Asp Asp Asp Asp Lys Val Pro Lys Asp Pro Val Trp Trp Asn Ser Ala
Enterokinase recognition site
EK cleavage site
EcoR V BstX I* Not I Xho I Xba I Apa I
1206 GAT ATC CAG CAC AGT GGC GGC CGC TCG AGT CTA GAG GGC CCG TTT AAA
Asp Ile Gln His Ser Gly Gly Arg Ser Ser Leu Glu Gly Pro Phe Lys
BGH reverse priming site
1254 CCC GCT GAT CAG CCT CGA CTG TGC CTT CTA GTT GCC AGC CAT CTG TTG
Pro Ala Asp Gln Pro Arg Leu Cys Leu Leu Val Ala Ser His Leu Leu
1302 TTT GCC CCT CCC CCG TGC CTT CCT TGA CCCTGGAAGG TGCCACTCCC
Phe Ala Pro Pro Pro Cys Leu Pro ***

Continued on next page

Cloning into pcDNA™4/HisMax A, B, and C, Continued

Multiple Cloning Site of Version C

Below is the multiple cloning site for pcDNA™4/HisMax C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotide indicates the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pcDNA™4/HisMax C is available for downloading from our website (www.invitrogen.com) or from **Technical Support** (see page 20).


T7 promoter/priming site
821 CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG
881 GGAGACCCAA GCTGGCTAGC GTTTAAACTT AAGCTTAGCG CAGAGGCTTG GGGCAGCCGA
QBI SP163 translational enhancer
941 GCGGCAGCCA GGCCCCGGCC CGGGCCTCGG TTCCAGAAGG GAGAGGAGCC CGCCAAGGCG
1001 CGCAAGAGAG CGGGCTGCCT CGCAGTCCGA GCCGGAGAGG GAGCGCGAGC CGCGCCGGCC
Polyhistidine Region
1061 CCGGACGGCC TCCGAAACC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT
Met Gly Gly Ser His His His His His His
Xpress™ Epitope
1110 GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC
Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr
Asp718 I Kpn I BamH I BstX I* EcoR I Pst I
1158 GAC GAT GAC GAT AAG GTA CCA GGA TCC AGT GTG GTG GAA TTC TGC AGA
Asp Asp Asp Asp Lys Val Pro Gly Ser Ser Val Val Glu Phe Cys Arg
Enterokinase recognition site
EK cleavage site
EcoR V BstX I* Not I Xho I Xba I Apa I
1206 TAT CCA GCA CAG TGG CGG CCG CTC GAG TCT AGA GGG CCC GTT TAA
Tyr Pro Ala Gln Trp Arg Pro Leu Glu Ser Arg Gly Pro Val ***
BGH reverse priming site
1251 ACCCGCTGAT CAGCCTCGAC TGTGCCTTCT AGTTGCCAGC CATCTGTTGT TTGCCCCTCC

Cloning into pcDNA™4/HisMax A, B, and C, Continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F´, DH5α) and select on LB plates containing 50-100 µg/mL ampicillin or 25-50 µg/mL Zeocin™ in Low Salt LB medium (see page 18). Select 10–20 clones and analyze for the presence and orientation of your insert.

Important

Any *E. coli* strain that contains the complete Tn*5* transposable element (i.e. DH5αF´IQ, SURE, SURE2) encodes the *ble* gene (bleomycin resistance gene). These strains will confer resistance to Zeocin™. For the most efficient selection, we recommend an *E. coli* strain that does not contain the Tn*5* gene (i.e., TOP10, DH5α, DH10, etc.).

We recommend that you sequence your construct with the T7 Forward and BGH Reverse primers to confirm that your gene is fused in frame with the N-terminal polyhistidine tag and the Xpress™ epitope. For ordering primers, see page 19. Note that if you use the T7 Forward primer to sequence your insert, approximately 300 bp of sequence encoding the QBI SP163 element and the N-terminal tag will precede the sequence of your insert.

Preparing a Glycerol Stock

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C in case you lose the glycerol stock.

1. Streak the original colony out on an LB plate containing 50 µg/mL ampicillin or 25 µg/mL Zeocin™ in Low Salt LB (see page 18). Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL ampicillin or 25 µg/mL Zeocin™.
3. Grow the culture to mid-log phase (OD600 = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Transfection and Analysis

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page 19 for ordering information).

Method of Transfection

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Invitrogen offers the Lipofectamine™ 2000 Reagent for mammalian transfection. Refer to our website (www.invitrogen.com) or call **Technical Support** (see page 20) for more information.

Positive Control

pcDNA™4/HisMax/*lacZ* is provided as a positive control vector for mammalian cell transfection and expression (see page 15) and may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the CMV promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed (see below).

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit and the β-Gal Staining Kit for fast and easy detection of β-galactosidase expression (see page 19).

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Detecting Fusion Proteins

The Anti-Xpress™ antibodies and the Anti-HisG antibodies are available from Invitrogen to detect expression of your fusion protein from pcDNA™4/HisMax (see page 19).

To detect the fusion protein by western blot, you need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.,* 24, 48, 72 hours, etc. after transfection). To lyse cells:

1. Wash cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 mL PBS and pellet the cells at 1,500 × *g* for 5 minutes.
3. Resuspend in 50 µL Cell Lysis Buffer (see page 18). Other cell lysis buffers are suitable.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.

   Note: You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 × *g* for 10 minutes to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration.

   Note: Do not use protein assays utilizing Coomassie® Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Note

The N-terminal peptide containing the Xpress™ epitope and the polyhistidine tag will add approximately 3.4 kDa to the size of your protein. Note that the QBI SP163 element is **not** translated.

Purification

You will need 5 × 10^6 to 1 × 10^7 **transfected** cells for purification of your protein on a 2 mL ProBond™ column (or other metal-chelating column). Refer to the manufacturer's instructions before attempting to purify your fusion protein. To prepare cells for lysis, refer to the protocol on page 12.

Creation of Stable Cell Lines

Introduction

pcDNA™4/HisMax vectors contain the Zeocin™ resistance gene for selection of stable cell lines using Zeocin™. We recommend that you test the sensitivity of your mammalian host cell to Zeocin™ as natural resistance varies among cell lines. General information and guidelines are provided below for your convenience. For more information about Zeocin™, refer to page 16.

Effect of Zeocin™ on Sensitive and Resistant Cells

The method of killing with Zeocin™ is quite different from neomycin and hygromycin. **Cells do not round up and detach from the plate.** Sensitive cells may exhibit the following morphological changes upon exposure to Zeocin™:

- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells)
- Abnormal cell shape
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and golgi apparatus, or other scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)
- Eventually, these "cells" will completely break down and only cellular debris will remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to cells not under selection with Zeocin™.

Selection in Mammalian Cell Lines

To generate a stable cell line expressing your protein, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected host cell line. In general, concentrations ranging from 50 to 1,000 µg/mL Zeocin™ are sufficient to kill the untransfected host cell line, with the average being 250 to 400 µg/mL. Test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Seed cells (20–25% confluent) for each time point and allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ (e.g., 0, 50, 100, 200, 400, 600, 800, and 1,000 µg/mL).
3. Replenish the selective medium every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth. Select the concentration that kills the majority of the cells in the desired number of days (4–10 days).

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Creation of Stable Cell Lines, Continued

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the gene of interest. The table below lists unique sites that may be used to linearize your construct prior to transfection. **Other restriction sites are possible. Note that for the enzymes listed below, the cleavage site is indicated for versions A, B, and C of pcDNA™4/HisMax.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp) (A,B,C)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Many
Mfe I	161	Upstream of CMV promoter	New England Biolabs
Nru I	208	Upstream of CMV promoter	Many
Bst1107 I	3063 (A), 3064 (B), 3062 (C)	End of SV40 poly A	AGS*, Fermentas, Takara, Boehringer-Mannhiem
Eam1105 I	4335 (A), 4336 (B), 4334 (C)	Ampicillin gene	AGS*, Fermentas, Takara
Fsp I	4557 (A), 4558 (B), 4556 (C)	Ampicillin gene	Many
Pvu I	4705 (A), 4706 (B), 4704 (C)	Ampicillin gene	Many
Sca I	4815 (A), 4816 (B), 4814 (C)	Ampicillin gene	Many
Ssp I	5139 (A), 5140 (B), 5138 (C)	Backbone	Many

*Angewandte Gentechnologie Systeme

Selection Tip

Some cells may be more resistant to Zeocin™ than others. If cells are dividing rapidly, Zeocin™ may not be effective at low concentrations. To overcome this resistance, we recommend that you place the cells at 4°C for 2 hours after plating (be sure to buffer the medium with HEPES). Then return the cells to 37°C. This stops the cell division process for a short time and allows Zeocin™ to act.

Continued on next page

Selecting Stable Integrants

Once the appropriate Zeocin™ concentration is determined, you can generate a stable cell line with your construct.

1. Transfect your cells using the appropriate protocol for your cell line. Include a sample of untransfected cells as a negative control.
2. After transfection, wash the cells once with 1X PBS and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent.
4. Replenish selective medium every 3–4 days until Zeocin™-resistant colonies are detected.
5. Pick and expand colonies in 96- or 48-well plates. Grow cells to near confluence before expanding to larger wells or plates.

Preparing Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™. You will need 5×10^6 to 1×10^7 cells for purification of your protein on a 2 mL ProBond™ column (see ProBond™ Purification System manual).

1. Seed cells in five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 240 × *g* for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 240 × *g* for 5 minutes. Remove PBS. You may lyse the cells immediately or freeze in liquid nitrogen and store at –80°C until needed.

Lysis of Cells

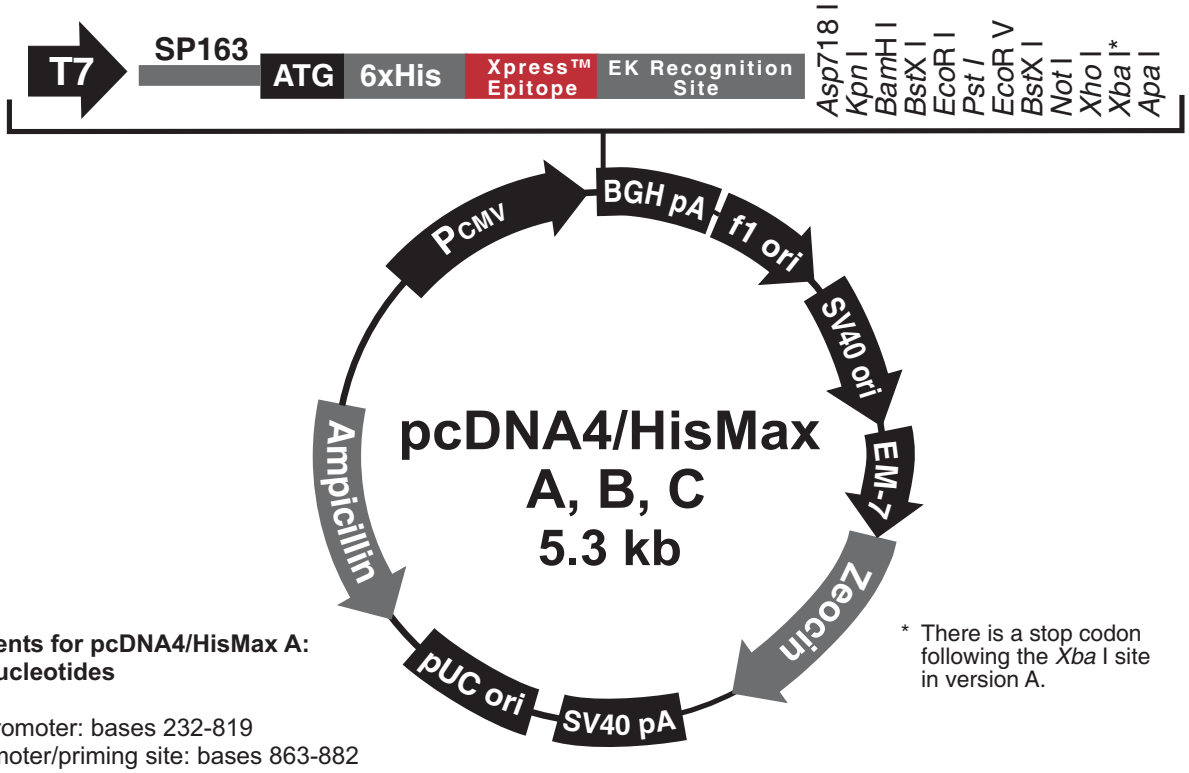
If you are using ProBond™ resin, refer to the ProBond™ Purification System manual for details about sample preparation for chromatography.
If you are using other metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

pcDNA™4/HisMax Vector

Map of pcDNA™4/HisMax

The figure below summarizes the features of the pcDNA™4/HisMax vectors. The sequences for pcDNA™4/HisMax A, B, and C are available for downloading from our website (www.invitrogen.com) or from **Technical Support** (see page 20).



* There is a stop codon following the *Xba* I site in version A.

Comments for pcDNA4/HisMax A:
5258 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
QBI SP163 translational enhancer: bases 917-1079
ATG initiation codon: bases 1080-1082
Polyhistidine tag: bases 1092-1109
Xpress™ epitope: bases 1149-1172
Enterokinase recognition site: bases 1158-1172
Multiple cloning site: bases 1172-1245
BGH reverse priming site: bases 1265-1282
BGH polyadenylation sequence: bases 1268-1495
f1 origin: bases 1541-1969
SV40 promoter and origin: bases 1996-2305
EM-7 promoter: bases 2353-2408
Zeocin™ resistance gene: bases 2427-2801
SV40 polyadenylation sequence: bases 2931-3061
pUC origin: bases 3444-4117
Ampicillin resistance gene: bases 4262-5122

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pcDNA™4/HisMax Vector, Continued

Features of pcDNA™4/HisMax

pcDNA™4/HisMax A (5258 bp), pcDNA™4/HisMax B (5259 bp), and pcDNA™4/HisMax C (5257 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert.
QBI SP163 translational enhancer	Increases expression of your recombinant protein via a cap-independent translation mechanism (Stein et al., 1998).
N-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™.
Xpress™ epitope tag	Allow detection of your recombinant protein with the Anti-Xpress™ Antibody.
Enterokinase cleavage site	Allows removal of the N-terminal tag from your recombinant protein using an enterokinase such as EnterokinaseMax™.
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in frame with the Xpress™ epitope and N-terminal polyhistidine tag.
BGH reverse priming site	Permits sequencing through the insert.
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992).
f1 origin	Allows rescue of single-stranded DNA.
SV40 early promoter and origin	Allows efficient, high-level expression of the Zeocin™ resistance gene in mammalian cells and episomal replication in cells expressing the SV40 large T antigen.
EM-7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the Zeocin™ resistance gene in *E. coli.*
Zeocin™ resistance gene	Selection of transformants in *E. coli* and stable transfectants in mammalian cells (Drocourt et al., 1990; Mulsant et al., 1988).
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA.
pUC origin	High-copy number replication and growth in *E. coli.*
Ampicillin resistance gene (β-lactamase)	Selection of transformants in *E. coli.*

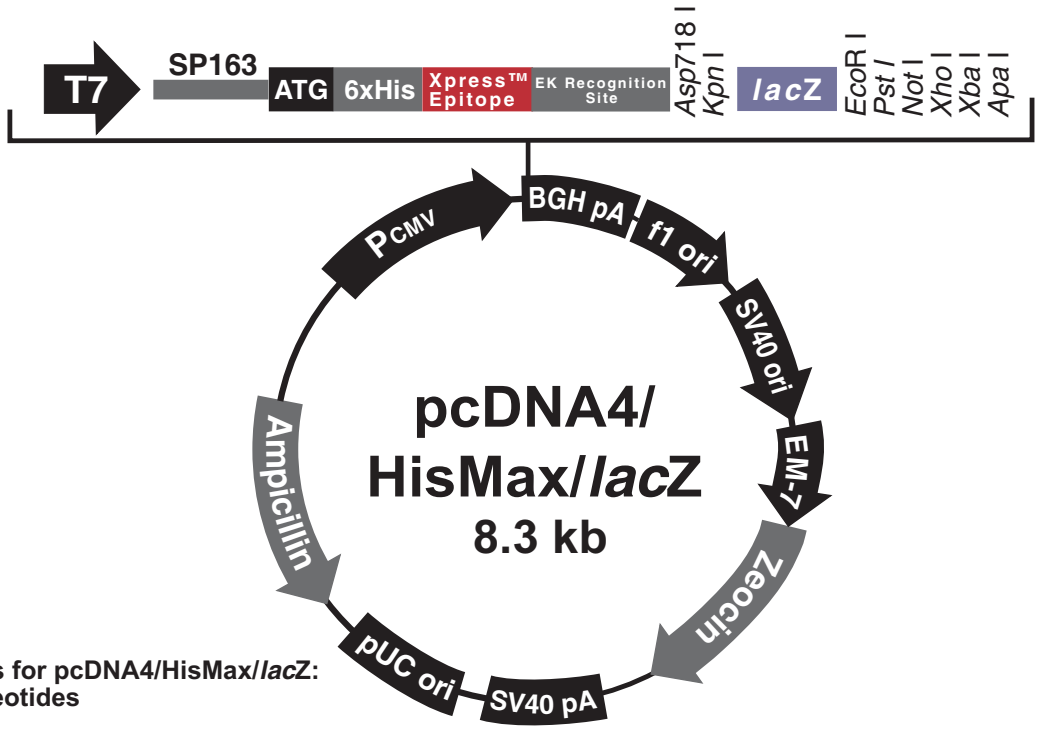
pcDNA™4/HisMax/*lacZ*

Description

pcDNA™4/HisMax/*lacZ* is a 8321 bp control vector containing the gene for β-galactosidase. This vector was constructed by ligating a 3.1 kb *Kpn* I-*Eco*R I fragment containing the *lacZ* gene into the *Kpn* I-*Eco*R I site of pcDNA™4/HisMax.

Map of Control Vector

The figure below summarizes the features of the pcDNA™4/HisMax/*lacZ* vector. The vector sequence for pcDNA™4/HisMax/lacZ is available for downloading from our website (www.invitrogen.com) or from **Technical Support** (see page 20).



Comments for pcDNA4/HisMax/*lacZ*: 8321 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
QBI SP163 translational enhancer: bases 917-1079
ATG initiation codon: bases 1080-1082
Polyhistidine tag: bases 1092-1109
Xpress™ epitope: bases 1149-1172
Enterokinase recognition site: bases 1158-1172
LacZ ORF: bases 1197-4247
BGH reverse priming site: bases 4328-4345
BGH polyadenylation sequence: bases 4331-4558
f1 origin: bases 4604-5032
SV40 promoter and origin: bases 5059-5368
EM-7 promoter: bases 5416-5471
Zeocin™ resistance gene: bases 5490-5864
SV40 polyadenylation sequence: bases 5994-6124
pUC origin: bases 6507-7180
Ampicillin resistance gene: bases 7325-8185

Zeocin™

Introduction

The pcDNA™4/HisMax vectors contain the Zeocin™ resistance gene for selection of stable cell lines using Zeocin™. We recommend that you test the sensitivity of your mammalian host cell to Zeocin™ as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

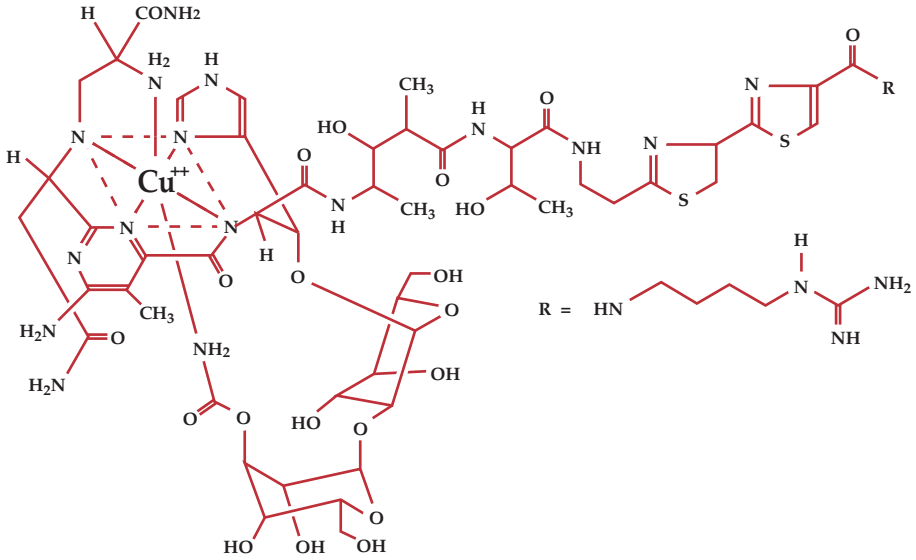
Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong anti-bacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula and Structure

The formula for Zeocin™ is $C_{55}H_{86}O_{21}N_{20}S_2Cu$-HCl and the molecular weight is 1,527.5 daltons. Zeocin™ is an HCl salt. The diagram below shows the structure of Zeocin™.



Continued on next page

Zeocin™, Continued

Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25-50 µg/mL in low salt LB medium* (see page 18 for recipe)
Mammalian Cells	50-1000 µg/mL (varies with cell line)

*Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (<90 mM).

Handling Zeocin™

- High salt and acidity or basicity inactivates Zeocin™. Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see page 18).
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store drug, plates, and medium containing drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Recipes

Low Salt LB Medium with Zeocin™

For Zeocin™ to be active, the salt concentration of the medium must be low (<90 mM) and the pH must be 7.5. For selection in *E. coli*, it is **imperative** that you prepare LB broth and plates using the following recipe. Note the lower salt content of this medium. Failure to use low salt LB medium will result in non-selection due to inactivation of the drug.

Low Salt LB Medium:
10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Thaw Zeocin™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/mL final concentration.
5. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Cell Lysis Buffer

50 mM Tris-HCl, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 mL, combine:

1 M Tris base	5 mL
5 M NaCl	3 mL
Nonidet P-40	1 mL

2. Bring the volume up to 90 mL with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 mL. Store at room temperature.

Note: Protease inhibitors may be added at the following concentrations:

1 mM PMSF
1 µg/mL pepstatin
1 µg/mL leupeptin

Accessory Products

Introduction

The following products may be used with the pcDNA™4/HisMax vectors. For details, visit www.invitrogen.com or contact **Technical Support** (page 20).

Item	Amount	Catalog no.
ProBond™ Purification System	6 × 2 mL precharged, prepacked ProBond™ resin columns and buffers for native and denaturing purification	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
Anti-Xpress™ Antibody	50 µL	R910-25
Electrocomp™ TOP10F´	5 × 80 µL	C665-55
One Shot® TOP10F´ (chemically competent cells)	21× 50 µL	C3030-03
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
β-Gal Assay Kit	80 mL	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
Zeocin™	1 gram	R250-01
	5 gram	R250-05
Lipofectamine™ 2000 Reagent	0.75 mL	11668-027
EnterokinaseMax™	250 units	E180-01

Primers

For your convenience, Invitrogen offers a custom primer synthesis service. Visit www.invitrogen.com for more details.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

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